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Enzymatic synthesis of DNA strands containing α-L-LNA (α-L-configured locked nucleic acid) thymine nucleotides

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Keywords: α-L-LNA, polymerase, enzymatic synthesis, modified nucleotide, triphosphate

Abbreviations: α-L-LNA, α-L-configured locked nucleic acid; HIV RT, human immunodeficiency virus reverse transcriptase; LNA, locked nucleic acid; PCR, polymerase chain reaction; DCM, dichloromethane; DEAE, diethylaminoethyl; HRMS, high resolution mass spectrometry; ESI, electrospray ionization; TBE, tris-borate-EDTA; EDTA, ethylenediaminetetraacetic acid; Dpo4, DNA polymerase IV; Tris, tris(hydroxymethyl)aminomethane

We describe the first enzymatic incorporation of an α-L-LNA nucleotide into an oligonucleotide. It was found that the 5′-triphosphate of α-L-LNA is a substrate for the DNA polymerases KOD, 9°N$_m$, Phusion and HIV RT. Three dispersed α-L-LNA thymine nucleotides can be incorporated into DNA strands by all four polymerases, but they were unable to perform consecutive incorporations of α-L-LNA nucleotides. In addition it was found that primer extension can be achieved using templates containing one α-L-LNA nucleotide.

Introduction

Locked nucleic acid[1-3] (LNA) and its diastereomer α-L-LNA[4-10] (α-L-configured locked nucleic acid) have found numerous applications within the field of nucleic acid chemical biology.[11-14] Recently, enzymatic incorporation of LNA nucleotides has been realized.[15-21] So far, no report on enzymatic incorporation of an α-L-LNA nucleotide, or any other LNA stereoisomeric nucleotide, has been published. In this paper we disclose the results of initial experiments on the compatibility of polymerases with α-L-LNA nucleotides.

As is the case with LNA, oligonucleotides containing α-L-LNA nucleotides show very efficient binding to complementary nucleic acids.[4-10] In addition, α-L-LNA nucleotides provide protection from nucleases when incorporated into oligonucleotides.[9,22,23] NMR studies concluded that DNA strands containing three α-L-LNA incorporations formed duplexes with DNA and RNA that were of the B-type and intermediate A/B-type, respectively.[24,25] Thus, α-L-LNA can be considered a DNA mimic.

The interesting properties of α-L-LNA led us to investigate the compatibility of polymerases with α-L-LNA nucleotides. We were encouraged by the fact that LNA nucleotides can be incorporated by a variety of polymerases.[15-21] In particular, KOD is very efficient at incorporating LNA nucleotides as well as reading LNA-containing templates.[18] In general, KOD was found to be non-restrictive with respect to both furanose ring puckering and C2'-modification since ribonucleotides could also be incorporated by KOD.[18]

The structures of LNA and α-L-LNA (**Fig. 1**) do not seem to be similar at first glance, but it has been shown that the positioning of atoms important for duplex formation (the O5' and O3' atoms of the sugar ring and the N1 atom of the nucleobase) overlay to a large extent in the two nucleotides.[4] Given the success of enzymatic incorporation of LNA nucleotides[15-21] we therefore speculated that the unusual sugar moiety of α-L-LNA could as well be accepted by some polymerases. Many examples of incorporation of nucleotides with unnatural sugar moieties exist in literature,[19,26-33] and a number of studies on stereoisomeric forms of 2'-deoxynucleotide triphosphates have shown that these in many cases act as chain terminators.[34-43]

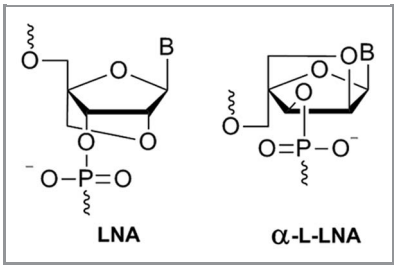

Figure 1. Structures of LNA and α-L-LNA nucleotides

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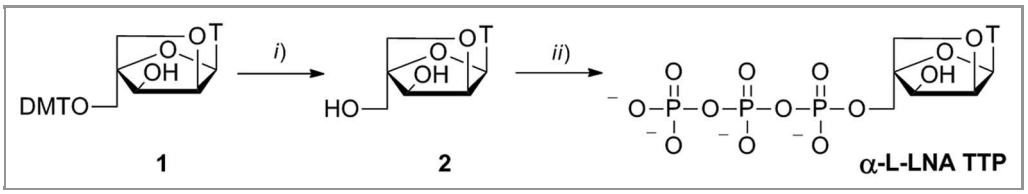

Scheme 1. Chemical synthesis of α-L-LNA TTP. (*i*) $Cl_2CHCOOH$, Et_3SiH, CH_2Cl_2 (100%); (*ii*) (1) $(MeO)_3PO$, proton sponge, $POCl_3$, -10°C → -5°C; (2) tri-*n*-butylamine, tributylammonium pyrophosphate, dimethylformamide, -5°C; (3) triethylammonium bicarbonate (4%).

Results

Synthesis of α-L-LNA TTP. The 5'-triphosphate of the thymine α-L-LNA nucleoside (α-L-LNA TTP) was synthesized in two steps from the known nucleoside 1 (**Scheme 1**).[7] Nucleoside 1 was detritylated and the resulting nucleoside 2[7] was converted to α-L-LNA TTP using the approach initially developed by Ludwig[44] and subsequently used by Veedu et al. to synthesize LNA NTPs.[16] This approach involves phosphorylation of the primary alcohol followed by reaction with pyrophosphate. α-L-LNA TTP was obtained in an overall yield of 4% after purification over an ion-exchange resin (see Materials and Methods for details).

Incorporation of α-L-LNA thymine nucleotides. Primer extension experiments were performed on three different primer-template complexes (**Fig. 2**) to test the limits of α-L-LNA-T nucleotide incorporation across 2'-deoxyadenosines in the template. Template T1 contained three 2'-deoxyadenosines surrounded by 2'-deoxynucleotides of the other three nucleobases, while template T2 contained eight consecutive 2'-deoxyadenosines. Template T3 contained only one 2'-deoxyadenosine for coding which was placed at the beginning. Thus, for T3 the polymerases needed to start by extending the primer with α-L-LNA-T as the first nucleotide.

Positive and negative control reactions were performed in parallel with reactions with α-L-LNA TTP. The reaction mixture of the positive controls contained all four natural dNTPs and led to extension of the primer to full length. Negative control reaction mixtures contained only dATP, dGTP and dCTP and were expected to stop at the first 2'-deoxyadenosine of the template. α-L-LNA incorporation was tested using reaction mixtures containing dATP, dGTP, dCTP and α-L-LNA TTP. Radiolabeled P1 and T1 were used as 19mer and 43mer markers.

The following seven polymerases were initially explored for their ability to incorporate α-L-LNA nucleotides: the Klenow fragment of *E. coli* DNA polymerase I (A-family polymerase); KOD, 9°N_m and Phusion DNA polymerases (B-family polymerase); human polymerase β (X-family polymerase); *S. solfataricus* DNA polymerase IV (Dpo4, Y-family polymerase); and HIV RT (reverse transcriptase family polymerase). It was found that the four most efficient polymerases for α-L-LNA nucleotide incorporation were KOD, 9°N_m, Phusion and HIV RT. **Figure 3** shows the results of primer extension experiments on the T1 template for these four polymerases. The experiment demonstrated that KOD, 9°N_m and Phusion DNA polymerases can efficiently accept α-L-LNA TTP as a substrate and afford the full-length extension products (lane 2). In particular, KOD is very quick at extending the primer to full length, however accompanied by some product degradation. Although HIV RT could also produce the fully extended product in low yield, the reaction did not progress to completion in the time the other three polymerases required.

Next, consecutive incorporation of α-L-LNA-T nucleotides was investigated (**Fig. 4**). KOD, Phusion and HIV RT were unable to extend the primer beyond the first incorporation of α-L-LNA-T (lane 2). 9°N_m DNA polymerase was able to incorporate consecutive α-L-LNA-T nucleotides, but full-length extension product was not observed. It appears that 9°N_m did not move forward methodically. Rather, 9°N_m seemed to quickly incorporate several α-L-LNA-T nucleotides before stopping extension. On comparison with a known DNA marker (not shown), we could conclude that a major product of the extension was 31 nucleotides long corresponding to consecutive incorporation of five α-L-LNA-T nucleotides, though some of the shorter products were also present in trace amounts.

P1	5'-^{32}P-TAATACGACTCACTATAGG-3'	19n
T1	3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'	43n
P1	5'-^{32}P-TAATACGACTCACTATAGG-3'	19n
T2	3'-ATTATGCTGAGTGATATCCGGGGCCCAAAAAAAATGGTCGCC-5'	42n
P1	5'-^{32}P-TAATACGACTCACTATAGG-3'	19n
T3	3'-ATTATGCTGAGTGATATCCATG-5'	22n

Figure 2. Primer-template complexes for primer extension experiments using α-L-LNA TTP. 2'-Deoxyadenosines encoding incorporation of α-L-LNA thymines are underlined.

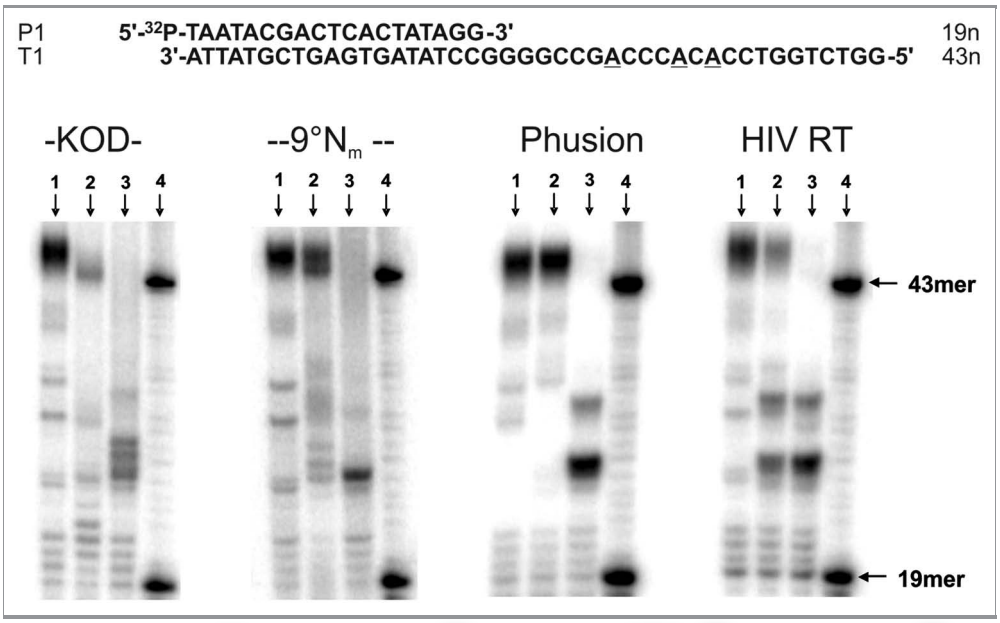

Figure 3. Primer extension using template T1. Lane 1: positive control (dATP, dGTP, dCTP and TTP); lane 2: incorporation of α-L-LNA-T nucleotides (dATP, dGTP, dCTP and α-L-LNA TTP); lane 3: negative control (dATP, dGTP and dCTP); lane 4: P1 and T1 (19mer and 43mer).

We also investigated whether the polymerases needed a running start in order to incorporate α-L-LNA-T nucleotides. We designed template T3 to direct the extension of the primer with α-L-LNA TTP as the first triphosphate to be used as substrate. The results in **Figure 5** show that KOD, 9°N_m and HIV RT were able to extend the primer to full length. In fact, KOD was so efficient with template T3 that misincorporation bands are seen in the positive and negative control reactions (**Fig. 5**, lanes 1 and 3). Phusion DNA polymerase proceeded with difficulty in extending the primer to afford only trace amounts of full-length product.

Primer extension using templates containing α-L-LNA nucleotides. Next, we investigated whether the four polymerases are able to use α-L-LNA TTP as substrate that can tolerate α-L-LNA nucleotides in the template. The commercially available T and 5-methyl-C α-L-LNA phosphoramidites were used to produce templates T4-T7 (**Fig. 6**). In templates T5 and T7, α-L-LNA nucleotides are placed one after another to produce a four

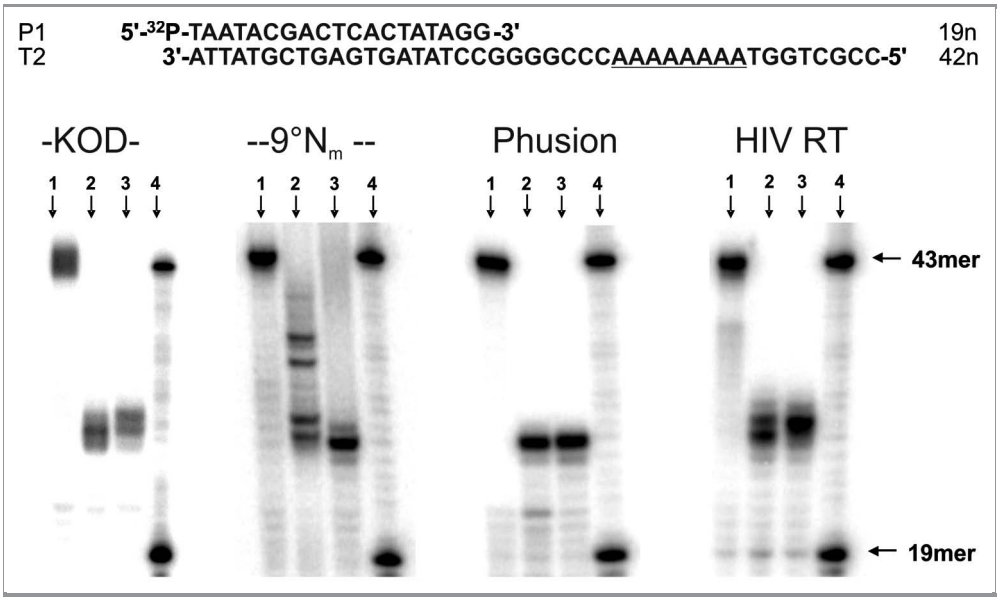

Figure 4. Primer extension using template T2. Lane 1: positive control (dATP, dGTP, dCTP and TTP); lane 2: incorporation of α-L-LNA-T nucleotides (dATP, dGTP, dCTP and α-L-LNA TTP); lane 3: negative control (dATP, dGTP and dCTP); lane 4: P1 and T1 (19mer and 43mer).

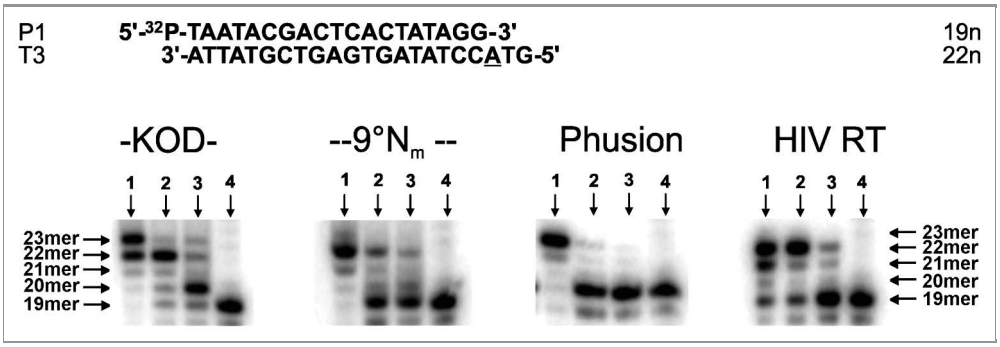

Figure 5. Primer extension using template T3. Lane 1: positive control (dATP, dGTP, dCTP and TTP); lane 2: incorporation of α-L-LNA-T nucleotides (dATP, dGTP, dCTP and α-L-LNA TTP); lane 3: negative control (dATP, dGTP and dCTP); lane 4: P1 (19mer).

nucleotide stretch of α-L-LNA nucleotides. In templates T4 and T6, α-L-LNA nucleotides are surrounded by 2'-deoxynucleotides.

Incorporation of 2'-deoxynucleotides using templates containing α-L-LNA nucleotides was tested by positive control reactions in which the mixture contained all four natural dNTPs. Negative control reactions were run in parallel. Negative control mixtures contained only dGTP, dCTP and dTTP (for incorporation across α-L-LNA-T) or dATP, dCTP and dTTP (for incorporation across α-L-LNA-5-methyl-C).

KOD, 9°N_m, Phusion and HIV RT which performed well at α-L-LNA-T incorporations were investigated for their ability to use templates containing α-L-LNA nucleotides. The results of primer extension experiments using templates T4-T7 are shown in **Figure** 7. All four polymerases demonstrated difficulties in extending the primer using templates T4-T7. However, template T6 which contained a single α-L-LNA-5-methyl-C nucleotide afforded the full-length extension product by all four polymerases (**Fig.** 7, lane 5) with KOD as the more efficient.

Discussion

Incorporation of α-L-LNA nucleotides. KOD, 9°N_m and Phusion DNA polymerases were able to incorporate α-L-LNA-T nucleotides when template T1 was used (**Fig.** 3) whereas incorporation using template T2 proved to be much more difficult (**Fig.** 4). A NMR structure determination of an α-L-LNA/DNA:DNA duplex concluded that the DNA backbone must rearrange to accommodate the α-L-LNA nucleotides in order for optimal Watson-Crick base pairing to take place.[24] This may explain the differences in primer extension between templates T1 and T2. In the case of T1, the growing primer strand did not contain consecutive α-L-LNA nucleotides, which meant that the backbone could possibly rearrange to keep the 3'-oxygen in the right position in the polymerase active site for further extension.

Primer extension using templates containing α-L-LNA nucleotides. In general, primer extension was difficult when using α-L-LNA-containing templates. Only the T6 template, which contained a single α-L-LNA-5-methyl-C nucleotide, was capable of templating full-length primer extension (**Fig.** 7). It was not surprising that templates T5 and T7 could not be used for primer extension since the polymerases had great difficulty in incorporating several α-L-LNA nucleotides in a row (**Fig.** 4). On this note it was, however, surprising that full-length extension product was not observed for template T4 since α-L-LNA nucleotides were not positioned consecutively. Possibly, rearrangement of the backbone induced by the α-L-LNA nucleotides is unfavorable for template function in general, e.g., because of steric clashes between with the polymerase and the

Figure 6. Primer-template complexes for primer extension experiments using templates containing α-L-LNA nucleotides. α-L-LNA nucleotides are underlined.

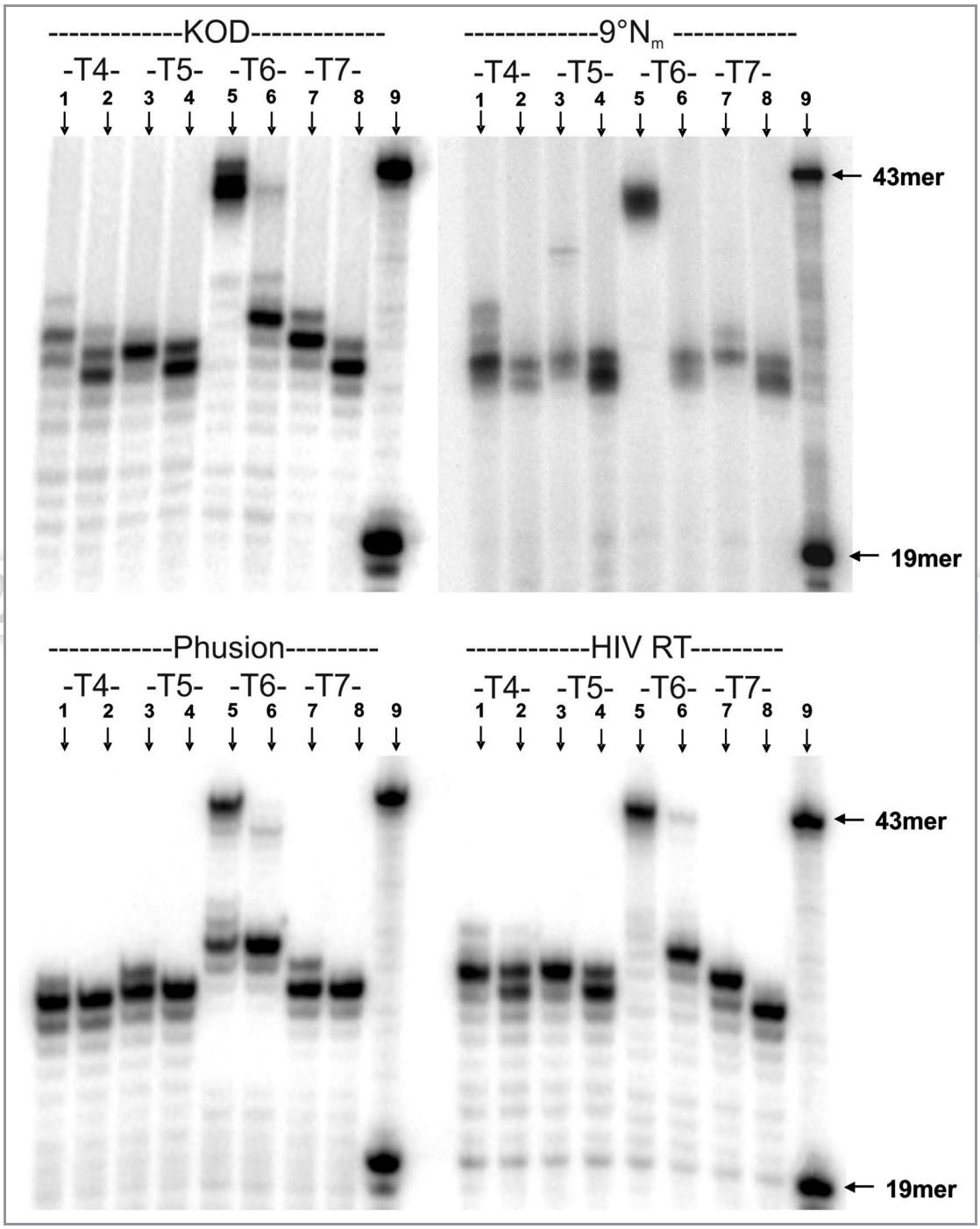

Figure 7. Primer extension using templates T4-T7. Lanes 1, 3, 5 and 7: positive controls (dATP, dGTP, dCTP and TTP); lanes 2, 4, 6 and 8: negative controls [dGTP, dCTP and dTTP (for incorporation across α-L-LNA-T); or dATP, dCTP and dTTP (for incorporation across α-L-LNA-5-methyl-C)]; lanes 1 and 2: template T4; lanes 3 and 4: template T5; lanes 5 and 6: template T6; lanes 7 and 8: template T7; lane 9: P1 and T1 (19mer and 43mer).

modified template strand. In general, primer extension was not halted at the position of the α-L-LNA nucleotide but rather at the subsequent position. Perhaps the high stability of the α-L-LNA: DNA pairing and/or the unique structure of the α-L-LNA nucleotides prevent the flexibility needed to position the primer strand 3'-oxygen correctly in the active site of the polymerases for further extension.

Comparison with LNA nucleotide incorporation. Of the seven polymerases tested for their ability to incorporate α-L-LNA nucleotides, the three B-family polymerases (KOD, 9°N$_m$ and Phusion) proved to be by far the most efficient. This is not to say that other B-family polymerases will be efficient too as Deep Vent and Therminator proved to be poor at incorporating LNA nucleotides, contrary to KOD, 9°N$_m$ and Phusion DNA polymerases.[18]

In general, incorporation of α-L-LNA nucleotides proved to be more difficult than incorporation of LNA nucleotides.[15-21] Furthermore, templates containing LNA nucleotides are readily used by KOD and 9°N$_m$ for primer extension reactions.[17,18] LNA nucleotides are RNA mimics and have been shown to conformationally steer the 3'-flanking sugar toward *N*-type sugar puckers.[45] In this light, it can be considered surprising that DNA-mimicking α-L-LNA nucleotides, which do not conformationally steer flanking nucleotide sugars,[24] are poorly accepted by polymerases when incorporated into template strands.

At present, KOD is the polymerase of choice for LNA nucleotide incorporation.[18,19,21] KOD also performed well with respect to α-L-LNA nucleotide incorporation though KOD could not incorporate consecutive α-L-LNA nucleotides. KOD can perform this task when using LNA triphosphates, and even extension of primers exclusively using LNA triphosphates has been achieved.[21] KOD has been shown to be non-restrictive in regard to sugar puckering and modification at the 2'-position.[18] Accordingly, α-L-LNA TTP was accepted as a substrate by KOD.

9°N$_m$ likewise performed well with respect to α-L-LNA nucleotide incorporation. 9°N$_m$ was the only polymerase capable of incorporating consecutive α-L-LNA nucleotides. 9°N$_m$ has also been used for incorporation of LNA nucleotides, and PCR amplification has been achieved using LNA triphosphates and this polymerase.[17]

Phusion was able to incorporate α-L-LNA nucleotides, but the primer could not be extended to full length when several α-L-LNA nucleotides in a row had to be incorporated. This was also observed for LNA incorporations, in which case Phusion was able to incorporate up to three consecutive LNA-T nucleotides.[16]

HIV RT was unsuccessful when we tried to incorporate LNA nucleotides into oligonucleotides (R. N. Veedu and J. Wengel, unpublished data), however, α-L-LNA nucleotides could be incorporated under certain conditions. Some full-length product was observed using template T1 and HIV RT was efficient at incorporating α-L-LNA nucleotides when template T3 was used. The incorporation profile of HIV RT was similar to the other three polymerases when templates containing α-L-LNA nucleotides were used in that full-length extension product was observed for template T6 only and for T4, T5 and T7, extension was halted at the first site of incorporation or possibly after a misincorporation.

Conclusion

KOD, 9°N$_m$, Phusion and HIV RT polymerases are able to accept α-L-LNA TTP as a substrate and to produce full-length primer extension reactions. However, primer extension involving consecutive incorporations of α-L-LNA nucleotides proved difficult. Templates containing more than one α-L-LNA nucleotide were not suitable for primer extension reaction when using these four polymerases. The fact that standard DNA polymerases are able to incorporate α-L-LNA nucleotides and to read α-L-LNA-containing templates is notable taking the highly unnatural conformational and configurational features of α-L-LNA nucleotides into consideration. Further advances are needed to obtain more efficient replication of α-L-LNA nucleotides, but the results presented herein represent the first step toward including α-L-LNA nucleotides in the context of biotechnology, e.g., aptamer evolution.

Materials and Methods

1-(2'-*O*,4'-*C*-methylene-α-L-ribofuranosyl)thymine (2) Nucleoside 1[7] (0.26 g and 0.45 mmol) was dissolved in methylene chloride (5.0 ml) and dichloroacetic acid (0.10 ml and 1.2 mmol) and Et_3SiH (0.15 ml, 0.94 mmol) were slowly added. The reaction was quenched after 1 h using MeOH. The mixture was evaporated to dryness. The product was obtained as a white solid after column chromatographic purification (0–5% MeOH/DCM, v/v). Yield 0.12 g (100%). ^{1}H NMR data were consistent with literature.[7] This known compound has been synthesized via a new route from the O5'-protected precursor.

The O5'-triphosphate of 1-(2'-*O*,4'-*C*-methylene-α-L-ribofuranosyl)thymine (α-L-LNA TTP) Nucleoside 2 (0.12 g, 0.45 mmol) was dissolved in $(MeO)_3PO$ (1.9 ml) and proton sponge (110 mg, 0.51 mmol) was added. The reaction mixture was cooled to -10°C and freshly distilled $POCl_3$ (44 µl, 0.48 mmol) was added dropwise under stirring. The mixture was stirred for 2 h at temperatures ranging from -10°C to -5°C. Bu_3N (0.32 ml, 1.3 mmol) and a 0.50 M solution of tributylammonium pyrophosphate in dimethylformamide (4.0 ml, 2.0 mmol) were added and the reaction mixture was stirred for another 2 h at -5°C. The reaction was quenched with a 0.50 M solution of triethylammonium bicarbonate (20 ml). The product was obtained after gravity column chromatographic purification using a WHATMAN DEAE cellulose-D50 anion-exchange resin and a gradient of triethylammonium bicarbonate in water. ^{31}P NMR (H_2O): δ -9.8 (γ-P), -10.1 (α-P), -22.4 (β-P). HRMS (ESI) *m/z* calculated for $C_{11}H_{16}N_2O_{15}P_3^-$ (M^-): 508.9768; found 508.9743.

General procedure for primer extension experiments. All water was distilled twice before use. Unmodified primers and templates were purchased from Sigma-Genosys. Templates containing α-L-LNA nucleotides were produced in-house using commercially available phosphoramidites (Exiqon). Primers were 5'-^{32}P labeled by [γ-^{32}P]-ATP (~6000 Ci/mmol, GE Healthcare) using T4 polynucleotide kinase (NEB, supplied by Medinova, M0201S) according to a procedure by the manufacturer. Primer and template were mixed in a 1:2 ratio. The mixture was heated to 80°C and subsequently slowly cooled to 37°C. Primer extension reactions were initiated by adding polymerase to a mix of buffer, nucleoside triphosphates and primer:template complex. The final concentration of nucleoside triphosphates in the used mix was approximately 190 µM. After a quick mixing, reaction tubes were incubated at the optimum temperature for the particular polymerase. Reaction volumes were 20.0 µl and a 5 µl aliqout was added to 2.25 µl loading buffer (95% formamide, 20 mM EDTA, bromophenol blue and xylene cyanol dyes) to stop the reaction. Products were separated on 13% 7 M urea polyacrylamide gels using TBE buffer (100 mM Tris, 90 mM boric acid, 1 mM EDTA, pH 8.4) and visualized by phosphor imaging.

Composition of the primer extension reactions.

KOD (TOYOBO, supplied by Novagen, 71085-3).

2.50 µl 10X KOD buffer 1
1.00 µl $MgCl_2$ (25 mM)
1.00 µl $MnCl_2$ (50 mM)
1.50 µl Triphosphate mixture (2.50 mM each)
0.60 µl Primer:template complex (5.0:10.0 µM)
0.60 µl KOD polymerase (2.50 U/µl)
12.8 µl Twice distilled water

9°N_m (NEB, supplied by Medinova, M0260S).

2.00 µl 10X Thermopol buffer
1.50 µl Triphosphate mixture (2.50 mM each)
0.60 µl Primer:template complex (5.0:10.0 µM)
0.60 µl 9°N_m polymerase (2000 U/ml)
15.3 µl Twice distilled water

Phusion (Finnzymes, F-530S).

4.00 µl 5X Phusion HF buffer
1.50 µl Triphosphate mixture (2.50 mM each)
0.60 µl Primer:template complex (5.0:10.0 µM)
0.60 µl Phusion (2000 U/ml)
13.3 µl Twice distilled water

Human polymerase β (Trevigen, 4020–500-EB).

2.00 µl 10X reaction buffer 8
1.50 µl Triphosphate mixture (2.50 mM each)
0.60 µl Primer:template complex (5.0:10.0 µM)
0.60 µl Human polymerase β (3.3 U/µl)
15.3 µl Twice distilled water

Klenow Fragment (Boehringer Mannheim; NEB, B7002S).

2.00 µl 10X buffer 2
1.50 µl Triphosphate mixture (2.50 mM each)
0.60 µl Primer:template complex (5.0:10.0 µM)
0.60 µl Klenow fragment (1.0 U/µl)
15.3 µl Twice distilled water

Dpo4 (TACS).

2.00 µl 10X Dpo4 buffer 15
2.00 µl $MgCl_2$ (100 mM)
1.00 µl $MnCl_2$ (50 mM)
1.50 µl Triphosphate mixture (2.50 mM each)
0.60 µl Primer:template complex (5.0:10.0 µM)
0.60 µl Dpo4 (0.40 µg/µl)
12.3 µl Twice distilled water

HIV RT (Worthington biochemical company, LS05003).

4.00 µl 5X HIV RT buffer (250 mM TRIS-HCl, 300 mM KCl, 12.5 mM $MgCl_2$)
1.50 µl Triphosphate mixture (2.50 mM each)
0.60 µl Primer:template complex (5.0:10.0 µM)
0.60 µl HIV RT (2.7 U/µl)
13.3 µl Twice distilled water

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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